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Collagen cross-links: location of pyridinoline in type I collagen

Nicholas Light* and Allen J. Bailey

AFRC Meat Research Institute, Langford, Bristol BS18 7DY, England

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Collagen from bone, dentine and tendon (type I), all of which contain the pyridinoline cross-link at varying levels, were each digested with CNBr. The resulting peptide mixtures were resolved by gel filtration on A1.5m agarose and assayed for pyridinoline. The polymeric cross-linked peptide complex, polyα1CB6 [(1980) Biochem. J. 189, 111] isolated from each of these tissues did not contain pyridinoline. Only one peptide fraction contained the pyridinoline cross-link; that identified as α2CB3,5. However, this peptide showed only a small increase in M_r in its cross-linked form (approx. 2000–5000) demonstrating that pyridinoline is not involved in the formation of polymeric structures like polyα1CB6. These data, considered in the light of the recent finding that pyridinoline is present in type I collagens from different sources in widely varying amounts, cast doubt on its role in collagen maturation.

Collagen *Cross-link* *Pyridinoline* *Peptide* *Tendon* *Bone*

1. INTRODUCTION

Newly formed collagen fibres are stabilised by divalent reducible cross-links derived from lysine and hydroxylysine [1,2]. Immature collagenous tissues vary in the proportion of these reducible keto-imine and aldimine cross-links, e.g., cartilage collagen contains the keto-imine, whilst dermal collagens possess the aldimine cross-link. During maturation the proportion of these keto-imine and aldimine cross-links falls to a low level [3] and we have proposed that they are intermediate cross-links that act as the precursors of the more complex stable cross-links of mature collagen [2]. As all collagens are initially cross-linked by these reducible bonds it might be expected that a common mechanism of age-related stabilisation exists resulting in the production of the 'mature' cross-links.

The nature of the 'mature' cross-links has been in dispute for some years. Several compounds have been described but no further support for them has been forthcoming. However, in 1977 Fujimoto and co-workers [4] isolated a fluorescent compound, named pyridinoline, from mature tendon. Although disputed at first [5] this compound has now been shown to be a cross-link of mature collagen [6–10]. Pyridinoline is formed through the interaction of 3 hydroxylysine residues by a mechanism which may proceed either via the reaction of two keto-imine cross-links [7] or by the reaction of the keto-imine with a hydroxylysine aldehyde [9].

In our approach to the identification of the mature cross-link we have demonstrated that the type I collagens in cornea, skin, tendon, bone and dentine are stabilised during maturation by a common pathway involving the formation of a complex polymeric matrix which links several molecules in the fibre [11,12]. In all cases the interactions appeared to proceed through the α1C-terminal peptide (α1CB6) and the small N-terminal

* Present address: Dorset Institute of Higher Education, Wallisdown Road, Wallisdown, Poole, Dorset, England

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peptides α1CB5 and α1CB1. Hence a common mechanism is apparent in type I collagen through the formation of the polymeric complex which we termed polyα1CB6. We showed by several criteria that this polymeric complex from mature collagen must contain the mature cross-link [13,14].

Here we have investigated the location of pyridinoline in type I collagens from bovine bone and tendon and present preliminary data which indicate that only the α2C-terminal peptide, α2CB3,5, contains pyridinoline.

2. MATERIALS AND METHODS

Biogel A1.5 was obtained from BioRad (High Wycombe, Bucks); DEAE-Sepharose 6B and Sephadex G-10 from Pharmacia (Uppsala, Sweden) and [^{3}H]lysine (399 mCi/mg) from the Radiochemical Centre (Amersham, Bucks). All chemicals and biochemicals used were analytical or HPLC grade and were obtained from BDH (Poole, Dorset) and Rathburn (Bucksburn, Scotland). Bovine skin, bone, dentine and achilles tendon were obtained fresh from mature animals at slaughter.

2.1. *Preparation of polyα1CB6 and other CNBr peptide fractions*

Bovine bone and dentine were splintered and decalcified exhaustively at 4°C in 0.25 M EDTA at pH 8.0. Tendons and skin were freed from adherent fat and were finely chopped. All tissues were water washed at 4°C and then suspended in 70% (v/v) formic acid at 20–30 mg/ml (wet wt). Polyα1CB6 was prepared after CNBr digestion on A1.5 m agarose in 1 M $CaCl_2$/0.05 M Tris-Cl, pH 7.5, and DEAE-Sepharose 6B as described in [13,14].

Other CNBr peptide fractions from bovine bone and tendon, particularly α2CB3,5, were prepared by A1.5 m agarose gel chromatography of CNBr peptide mixtures. In the case of bovine tendon all of the CNBr peptide material was pooled in several fractions from an A1.5 m column developed in 1 M $CaCl_2$/0.05 M Tris-Cl, pH 7.5. Each fraction was dialysed at 4°C in the dark against 0.1 M acetic acid and the freeze-dried peptide material was assayed for pyridinoline after acid hydrolysis. Crude α2CB3,5 obtained from mature bovine tendon by A1.5 m agarose gel filtration was further purified by high-performance liquid chromatography (HPLC) on a 25 × 0.46 cm Bakerbond C18, 33 nm pore-size reverse-phase column as described [15]. Finally, 7 mg of the peptide fraction was chromatographed on a column (7 × 1.6 cm) of CM-Sepharose 6B in 0.2 M sodium citrate buffer, pH 3.6, essentially as in [12].

2.2. *Acid hydrolysis and amino acid analysis*

Samples of polyα1CB6 from bovine tendon, dentine and bone were taken up at 1 mg/ml in 6 M HCl and hydrolysed in sealed tubes at 110°C for 24 h. Hydrolysates were rotary evaporated to dryness at 50°C, taken up in 0.001 M HCl and stored in darkness at −20°C. All samples were analysed as quickly as possible after production of peptides and always within 1 week.

Hydrolysates were assayed for pyridinoline as follows. First, hydrolysates were chromatographed on Sephadex G-10 (95 × 1.6 cm) in 0.05 M acetic acid. 2-ml fractions were collected and assayed for fluorescence at 400 nm after excitation at 295 nm in a Perkin-Elmer 3000 fluorimeter. Sephadex columns were calibrated with M_r markers including pyridinoline (M_r 450) made by the method of Fujimoto et al. [4]. The fluorescent fractions with M_r 400–500 were pooled and subjected to a single column amino acid analysis on a Jeol amino acid analyser in citrate buffers. Pyridinoline was identified by its elution position and fluorescent characteristics [4].

2.3. *Pyridinoline content of α2CB3,5 peptide*

A portion of the purified α2CB3,5 material from mature bovine tendon was dissolved in 0.5 M acetic acid and its fluorescence emission spectrum was obtained on excitation at 295 nm. This material was retained as a control whilst a second portion of the peptide was dissolved in 0.5 M ammonium bicarbonate, pH 9.5. The emission spectrum of this sample was obtained with excitation at 325 nm. Both samples were then heated at 37°C in the dark for 18 h. After incubation the samples were dialysed at 4°C in the dark against excess water prior to freeze drying and analysis by SDS-polyacrylamide gel electrophoresis as in [11].

3. RESULTS

3.1. *Analysis of polyα1CB6 for pyridinoline*

No trace of pyridinoline was found in hydrolysates of polyα1CB6 from bovine skin, tendon, bone or dentine by the criteria described in section 2. These techniques proved more than adequate to analyse and quantify pyridinoline in very small amounts of tissue (e.g., cartilage preparations) or peptides containing the compound. Even when 20 mg polyα1CB6 was analysed (in which, at a level of 1 mol cross-link/mol polyα1CB6 the expected yield would be in excess of 500 nmol) we could not detect pyridinoline.

3.2. *Pyridinoline in CNBr peptide fractions*

Fig.1 shows a typical elution profile for CNBr digested bovine tendon collagen from the A1.5 m agarose column. Analysis of the fractions for fluorescence at 400 nm showed that all peptide material was positive when an excitation beam at 295 nm was used. The fractions indicated in fig.1a were pooled and a portion of each was dried after dialysis and then hydrolysed in 6 M HCl.

When these acid hydrolysates of separated CNBr peptides were analysed for fluorescence after gel filtration on Sephadex G-10 (fig.1b) only the fraction containing α2CB3,5 showed a fluorescent peak at M_r 450 (the reported M_r of pyridinoline, see [4]). This fraction was pooled (fig.1b) and shown to be pyridinoline by its position on the amino acid analyser and by its excitation and emission spectra at pH 3.0 and 10.0 as described [4].

3.3. *Properties of purified α2CB3,5*

α2CB3,5 peptide was purified from the crude fraction 2 shown in fig.1a by HPLC and ion-exchange chromatography (fig.2a,b). The peptide chromatographed as a single peak in both instances and it appeared that this peak was relatively homogeneous with respect to α2CB3,5. The yield of the final product, assessed for purity by SDS-polyacrylamide gel electrophoresis, was approx. 40%, most losses occurring in the ion-exchange step.

Analysis of pure α2CB3,5 for fluorescence at excitation and emission wavelengths of 295 and 400 nm, respectively, showed very similar acid/alkali fluorescence spectra to those described for free pyridinoline [4]. Also, when the pure α2CB3,5 from mature bovine tendon was treated under mild alkaline conditions (which are known

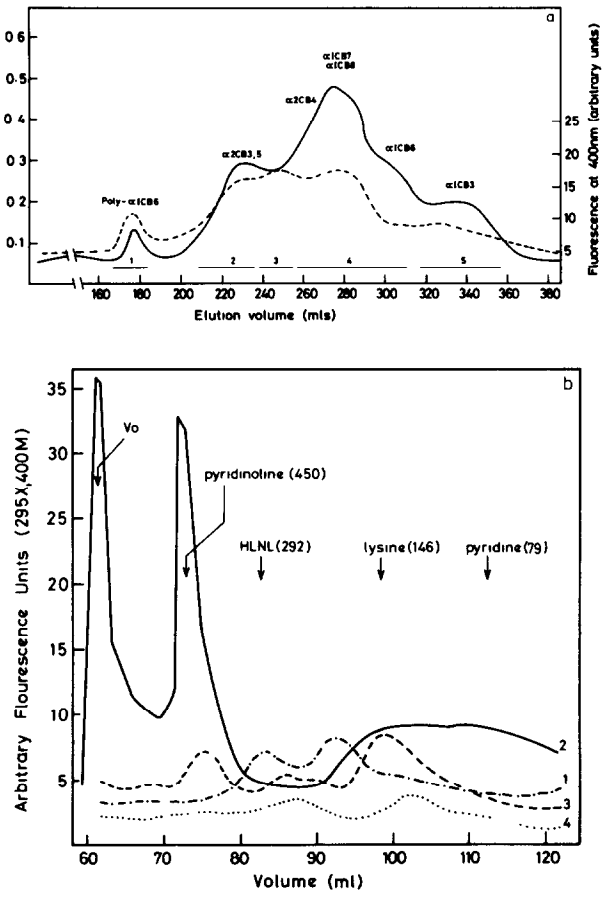


Fig.1. (a) Separation of CNBr peptides of bovine tendon type I collagen by gel filtration on agarose A1.5 m. CNBr peptides of bovine tendon type I collagen were separated in 1 M $CaCl_2$/0.05 M Tris-Cl, pH 7.5, on agarose A1.5 m was described in section 2. The effluent was monitored at 230 nm and for fluorescence at 400 nm with excitation at 295 nm after acidifying each fraction. Fractions were pooled as shown by the bars. (——) Absorbance at 230 nm, (– – –) fluorescence at 400 nm. (b) Pyridinoline content of CNBr peptide fractions derived from A1.5 m gel filtration. Peptide fractions indicated by the bars in panel a were pooled, dialysed, freeze-dried and hydrolysed in 6 M HCl. The hydrolysates were each subjected to gel filtration on Sephadex G-10 in 0.05 M acetic acid and the effluents were monitored for fluorescence at 400 nm with excitation at 295 nm. HLNL, hydroxylysinonorleucine (M_r 292).

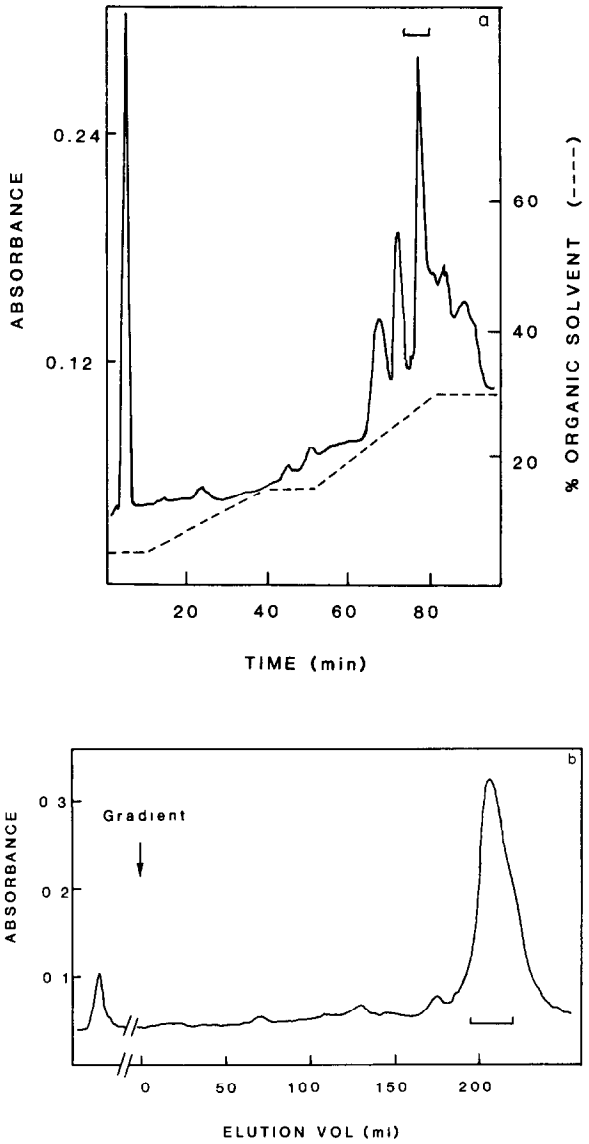


Fig.2. Purification of α2CB3,5 peptide from mature bovine tendon. (a) The crude fraction 1 from fig.1 was subjected to reverse-phase HPLC on a 25 × 0.46 cm Bakerbond 33 nm pore-size C18 column at 55°C and a flow rate of 1 ml/min. The column was equilibrated in a solvent containing 0.05 M ammonium bicarbonate and 4% (v/v) trifluoroacetic acid and peptides were eluted with a gradient of tetrahydrofuran as described in [15]. The bar denotes the α2CB3,5-rich fraction pooled. (b) α2CB3,5 peptide pooled as shown in panel a was further purified by ion-exchange chromatography on CM-Sepharose 6B in 0.2 M sodium citrate buffer under conditions described in [12]. The pure peptide was pooled as indicated by the bar.

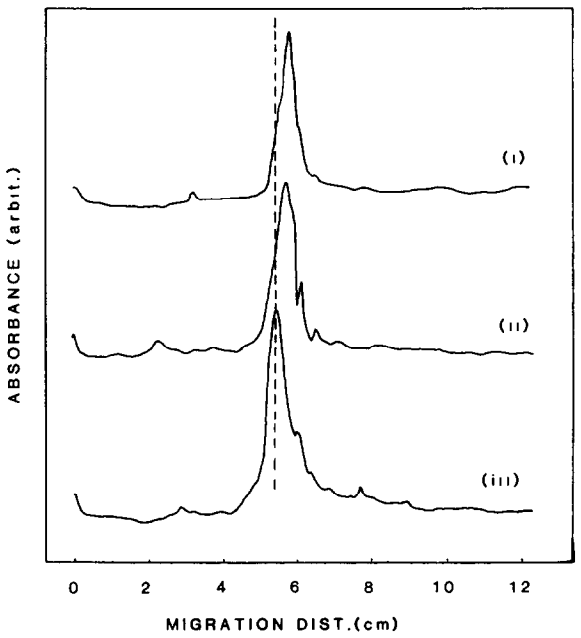


Fig.3. Densitometric scans of SDS-polyacrylamide gels. Densitometric scans of SDS-polyacrylamide gel electrophoretograms of α2CB3,5 peptide fractions from soluble bovine tendon collagen (i), mature bovine tendon collagen after treatment at pH 9.5 and 37°C for 18 h (ii), and from mature bovine tendon but without any treatment prior to analysis (iii).

to cleave pyridinoline, see [17]) the major peptide product migrated on SDS-polyacrylamide gels with a slightly greater mobility than untreated α2CB3,5 indicating the loss of one or more small peptides (fig.3). This 'hydrolysed' product was identical to α2CB2,5 prepared from soluble type I collagen and contained no pyridinoline fluorescence (fig.3).

4. DISCUSSION

The hydroxylysine-derived collagen compound pyridinoline has been widely accepted as the so-called mature cross-link of collagen; i.e., the cross-linking moiety responsible for the increased stability of mature collagen [18]. We suggest however, that consideration of the available data in the literature and the results of the present study highlight certain anomalies concerning this compound which make a re-evaluation of its proposed role essential.

Eyre et al. [18] assayed pyridinoline in various

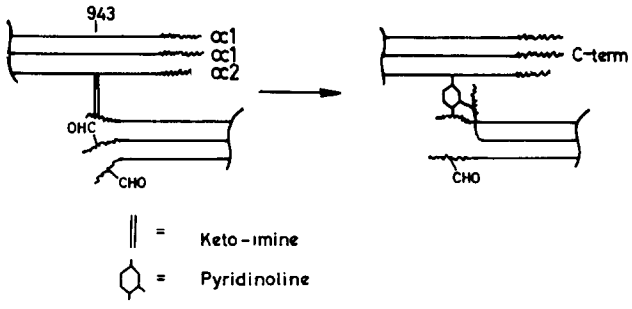
tissues and reported values of 1–2.6 residues/mol collagen in cartilage, 0.4 residue/mol collagen in tendon, 0.1–0.2 residue/mol collagen in bone and not detectable in skin collagen. The compound is known to be formed from 3 hydroxylysine residues so the values for bone are surprisingly low considering the high hydroxylation of the tissue and suggest that the role of pyridinoline may be different in the various tissues in which it has been located. More recent studies have shown that type IV collagen, which is cross-linked only by the keto-imine cross-link in young tissue, does not contain pyridinoline [19]. On the basis of these results one would have to predict different mechanisms of stabilisation via pyridinoline for type II collagen, the type I collagens in skin, bone and tendon and for type IV collagen. Such diversity seems surprising and leads to the speculation that other, as yet undiscovered, mechanisms of stabilisation may exist which are common to all the genetic forms of collagen.

Another anomaly arising from the data presented here is as follows. We have shown that type I collagens from several sources are cross-linked in the mature state through a polymeric network which may involve up to 10 molecules at a time. A complex polymeric peptide can be derived from this insoluble collagen by CNBr digestion and we have termed this material poly-α1CB6 [13,14]. This material has been shown by several criteria to contain heat- and acid-stable cross-links [13] but careful analysis of the peptide complex from skin, tendon, bone and dentine in the present study showed that pyridinoline was not present. This is confusing as Fujimoto purified a peptide from a thermolysin digest of mature collagen which he reported to contain pyridinoline linking two partial α1CB6 sequences and one α1CB5 sequence based on amino acid analysis only [20]. This paradox can be resolved, however, if we consider the two peptides α1CB6 and α2CB3,5, the C-terminal peptides of the α1 and α2 chains, respectively. Both peptides contain tyrosine and histidine among other residues uncharacteristic of the rest of the collagen molecule. Given that the major sequences of the α1 and α2 chains are very similar due to the Gly-Pro-X repeat common to both, these similarities could explain the misinterpretation of Fujimoto's amino acid analysis data. In other words, Fujimoto's thermolysin peptide [20] could have been derived from α2CB3,5 rather than α1CB6.

In support of this hypothesis we have now isolated and partially purified α2CB3,5 peptide from type I collagen CNBr digests. When obtained from mature tissues this peptide is the only one derived from type I collagen which does contain pyridinoline. Furthermore, the peptide, in cross-linked form, has only a slightly elevated M_r compared to the peptide prepared from soluble collagen (uncross-linked) indicating that it is cross-linked to very small peptides in the collagen molecule.

As Sakura and Fujimoto [17] showed that pyridinoline is rapidly broken down by alkaline conditions we incubated α2CB3,5 containing pyridinoline at pH 9.5 in an attempt to cleave the cross-link and yield uncross-linked α2CB3,5. SDS-polyacrylamide gel analysis of the purified peptide before and after alkali treatment confirmed that the α2CB3,5 had been cross-linked by pyridinoline to other small peptides. Amino acid analysis of the treated peptide also confirmed that all of the pyridinoline had been destroyed by the mild alkali conditions.

Robins and Duncan [9] have proposed a reaction scheme for the formation of pyridinoline in type II cartilage collagen which indicates that the cross-link only links two collagen molecules. Based on our evidence for the presence of pyridinoline in α2CB3,5 a similar mechanism of formation can be envisaged in type I collagen. We suggest that since the C-terminal non-helical sequence of the α2 chain lacks lysine, pyridinoline must be formed from condensation of the hydroxylysine aldehyde in the N-terminal non-helical region with the pre-existing keto-imine cross-link between a N-terminal non-helical sequence of the same molecule and the α2CB3,5 helical region of an adjacent molecule.



Pyridinoline would therefore only cross-link two collagen molecules. Significantly, in this study we showed that pyridinoline in type I collagen was associated only with non-polymeric α2CB3,5 and would thus be unlikely to bring about lateral cross-linking on the scale needed to induce the age-related changes in collagen seen on maturation [21] as would the much larger polyα1CB6 complex.

Critical review of the literature and the data in this report, therefore, shows that pyridinoline is present in highly hydroxylated collagens in variable amounts which may not be consistent with its proposed role as the stabilising cross-link of mature collagen. Indeed, two major tissues, skin, which is not highly hydroxylated, and type IV collagen, which is, both completely lack the moiety. Evidently, pyridinoline cannot be the only mature cross-link of collagen. This is corroborated by our study of poly-α1CB6 which is known to account for the stabilisation of type I collagen on maturation yet contains no pyridinoline. Further, examination of the location of pyridinoline both in type I and type II collagens ([9] and this report) has led to the conclusion that the cross-link may only link two molecules thus being an age-related intramolecular bond. In the light of these findings there is an evident need for further, far more detailed studies of the location and role of this intriguing compound of collagen.

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